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We have conceived of an approach to prepare novel compounds for the estrogen receptor that might be useful in the imaging and diagnosis of breast cancer, by the creation of steroidal-based imaging agents which take advantage of the desirable properties of gallium as a radioisotope..

We have made good progress on the preparation of the carbon skeletons needed to assemble these compounds. Synthetic routes to those compounds functionalized in the steroid D-ring have been developed, as well as a carbocyclic analog of the compounds designed to mimic the benzestrol class of estrogens.

Future efforts will be directed at completing the synthesis of the gallium-containing compounds of the D-ring class, which appear to be the most promising, and preparation of these compounds in radioactive form.

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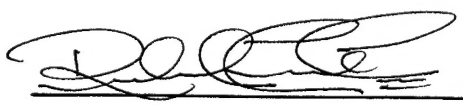

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TABLE OF CONTENTS

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PRINCIPAL INVESTIGATOR: Richard R. Cesati III

TITLE: Gallium-Containing Estrogens as Receptor-Based Breast Tumor Imaging Agents

ORGANIZATION: University Of Illinois

INTRODUCTION AND GOALS OF THE PROJECT

Radioisotopes of gallium, as the trication Ga^{3+}, are regularly used for medical imaging, and other gallium compounds are being investigated for their potential as radiopharmaceuticals.[1-3] These radiodiagnostic agents consist of ligands chelated to either ^{67}Ga or ^{68}Ga radioisotopes.[4-6] Since the ^{66}Ga and ^{68}Ga isotopes are positron-emitting nuclides with convenient half-lives, we became interested in incorporating gallium into estrogens for imaging of breast tumors by PET.

Breast tumors can be imaged by the binding of appropriately labeled estrogens to the estrogen receptor (ER), which is found in many tumors, and these images provide useful prognostic information concerning cancer stage and tumor responsiveness to hormone therapy.[4,7-12] So far, however, breast tumor imaging through the ER has been accomplished only with a number of radiohalogen-labeled estrogens. The availability of the halogen radionuclides needed to produce these ER-binding agents, however, is limited, either by their short half-lives (e.g., for ^{18}F, $t_{1/2}$ = 110 min) or by difficulties in their production (e.g., production of ^{123}I requires high energy cyclotrons). Because ^{66}Ga and ^{67}Ga have relatively long half-lives ($t_{1/2}$ = 9.4 h and 3.3 days, respectively) and ^{68}Ga ($t_{1/2}$ = 68 min) is available from a long-lived ^{68}Ge ($t_{1/2}$ = 288 days) generator, replacing the radiohalogen in these imaging agents with these gallium radionuclides would make them more widely available and might also simplify their preparation.

When gallium is used as the citrate salt of the Ga^{3+} cation, most *in vivo* transport and uptake of this cation is mediated by iron-binding proteins, principally transferrin, because of the similar ionic radii of Ga^{3+} and Fe^{3+}.[13] If undesirable for a given procedure, binding of Ga^{3+} to transferrin can be prevented by the use of high-affinity multidentate ligands, generally hexadentate ones.[14] An alternative form of gallium that has been evaluated for radiopharmaceutical purposes is $(CH_3)_2Ga^+$, a cation in which the metal-carbon bonds are highly stable toward hydrolysis.[1,15] The charge and size of this cation apparently make it sufficiently dissimilar to Fe^{3+} to prevent

significant binding by plasma proteins.[15] Low affinity for plasma proteins, a tetracoordinate (rather than hexacoordinate) geometry, and the alkyl ligand environment of the metal suggest that dimethylgallium compounds may be more adaptable to the design of ligands for biological receptors than are multidentate Ga^{3+} complexes.

The highly stable metal-carbon bonds in dimethylgallium hints at further elaboration into actual steroidal and non-steroidal skeletons whereby a single gallium atom would cause minimal perturbation of the estrogenic framework of high affinity ligands. These chemical properties, in combination with the desirable properties of gallium as a radiolabel, should provide an effective means of imaging ER^+ tumors.

BODY

Experimental Approach

Considerations in the Design of Gallium Compounds that Mimic ER Ligands

The estrogen template outlined in Figure 1 indicates that the gallium-containing estrogens should mimic the estradiol molecule or include a bis-phenolic structure. Within this framework, there are two methods of characterizing these estrogens: by carbon skeleton (steroidal or nonsteroidal), and by metal incorporation site (conjugated or integrated). Conjugate structures consist of a preformed active estrogen to which a metal fragment (moiety) is attached or "conjugated" usually by an alkyl spacer. In an "integrated" design, the metal is actually part of the molecular framework that gives the estrogen its affinity for ER.

In the original proposal we intended to systematically vary the combinations of the estradiol structure, using the integrated design, in conjunction with variations in the gallium moiety to produce the representative gallium-containing estrogens shown in Figure 1. Schemes 1 and 2 detail the well precedented synthetic routes to most of the target structures. The transformations involving gallium also have strong literature precedent, although novel gallium chemistry may need to be developed, especially at the tracer level.

The design of the estradiol mimics was based on the following criteria: (1) known high affinity for ER; (2) steric tolerance of substituents, (7α, 14α and 17α); and (3) ease of elaboration into know gallium chemistry. The topological similarity of gallium and carbon, with respect to coordination geometry, is a key feature in "disguising" the radiolabel in this design: another desirable feature of the integrated design (Table 1).

The gallium moieties presented in Figure 1 were selected, as well, for their anticipated kinetic and/or thermodynamic stability on the basis of literature precedent as well as previous

results from our laboratory. The use of bis-Grignard reagents in the preparation of six-membered gallocycles has extensive precedent, as does the use of intramolecular chelation as a means of stabilization of the gallium. In the original proposal, some reservations in the use of these bis-Grignard reagents, (possible side-reactions due to intermolecular cross-coupling, cyclization and polymerization), was presented, however, models studies to date indicate that these systems should behave as desired.

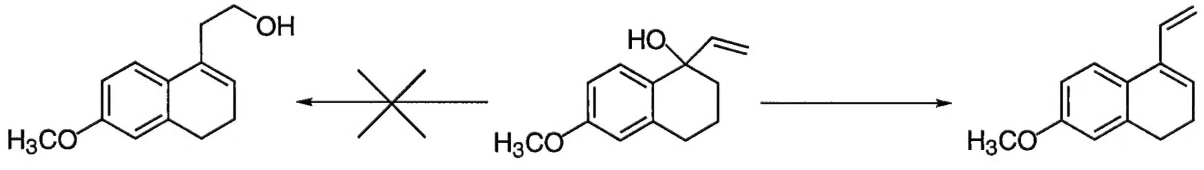
In vivo dissociation of a gallium moiety from the molecular frameworks shown in Figure 1 may still present a problem, despite the design efforts to maximize stability. Hydrolytic instability could be counteracted with structural modifications that stabilize gallium's coordination environment. Chelating N,N-dialkylamino groups are known to improve the stability of four-coordinate compounds toward hydrolysis,[16] and this fact could guide the modification or redesign of the targeted compounds. Another possible approach that is not developed in Figure 1 is to place gallium in a chelating amino acid moiety such as glycinatodimethylgallium.[17] In any case, extra tuning of a compound's properties could be achieved with electron-withdrawing groups or electron-donating groups that change the Lewis basicity of the ligand for gallium.

As we discuss below, we have made good progress in the syntheses of compounds with the steroidal design, as well as obtained a good lead into the non-steroidal class.

Results and Discussion

Synthesis of Class IA: Integrated Steroids

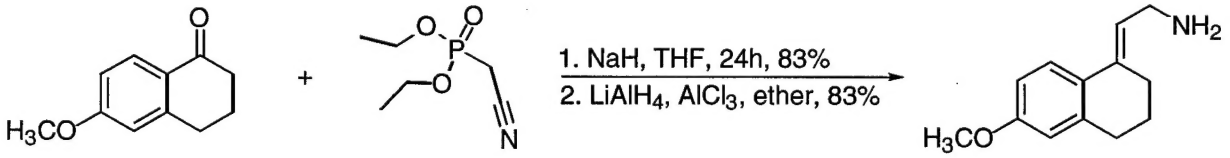
The synthetic approach outlined in Scheme 1 in the original proposal proved to be an unsuccessful one. We were unable to affect the acid catalyzed rearrangement of the allylic alcohol **2** to the homoallylic alcohol **3** without obtaining significant dehydration to the corresponding diene. Although, this diene could undergo hydroboration and oxidation to the desired alcohol **3**, an alternative route was devised.



Using tetralone **1**, ethyl lithioacetate could be added to obtain the β-hydroxy alcohol. This alcohol could then be dehydrated to yield a mixture of the α,β and β,γ-unsaturated esters. The β,γ ester could be obtained selectively from this mixture by subsequent α-lithiation and quenching

under kinetic conditions. Standard reduction of the ester afforded the homoallylic alcohol **3**. Swern-type oxidation of **3** afforded the aldehyde **4**, not as the desired β,γ-isomer, but rather almost exclusively as the undesired α,β-isomer. Reductive amination of this unsaturated aldehyde was therefore not attempted.

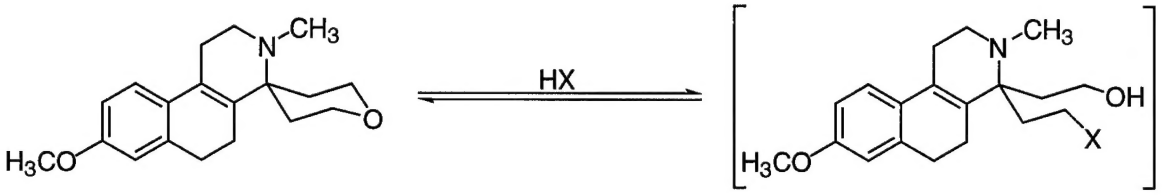
The successful use of the ethyl lithioacetate above prompted us to investigate other acetyl nucleophiles. Lithioacetonitrile provided not only an excellent nucleophile for the two-carbon homologation, but rather, more importantly, provided facile access to the amine precursor **5**. Rather than use lithioacetonitrile, which lead to a mixture of olefins as above, we settled on using diethyl cyanomethylphophonate in a Horner-Emmons reaction, which provided exclusively the α,β-unsaturated nitrile in good yield. Successful reduction of the nitrile was then achieved using equimolar amounts of lithium aluminum hydride and aluminum chloride.[18]



Scheme 1 suggests the use of a secondary amine in the Pictet-Spengler reaction. However, we found, through the literature and preliminary studies of model systems, that secondary amines react more slowly than primary amines in Pictet-Spengler cyclizations: reactions of ketones with secondary amines are very rare. Although standard conditions for the formation of piperidines were successful for several aliphatic and aromatic ketones and aldehydes, we were initially unable to obtain cyclization with diethyl acetonedicarboxylate.

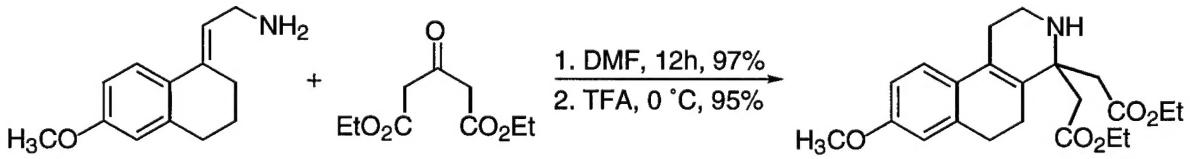
We were able to obtain successful cyclization with diethyl ketomalonate. We hypothesized that methylation and single carbon homologation of this compound would provide us with the ester of compound **7**. However, several attempts were made to homologate this compound, to the desired β-amino ester, without success.

Cyclization was also successful with tetrahydro-4*H*-pyran-4-one. This product was important in that it provide us with all of the carbons of the final product. Ring opening and halogenation of the pyran was all that was needed to obtain precursor **9**. Several attempts were made to open the pyran, without success: the pyran appeared to be stable under the most vigorous of conditions. It was not until we obtained the diol **8** independently that we realized the conditions necessary for the cleavage of the pyran were ultimately leading to cyclization of the ring-opened products.



The conditions used in the Pictet-Spengler reaction above, always provided mixtures of olefin isomers: exo and endo with respect to the tetrahydronaphthalene ring. Although these mixtures could be biased toward the desired endo product with extended heating times, separation of the minor isomer was usually required, and often tedious. Therefore, we sought to obtain a milder method of cyclization.

We found that the amine could be reacted under mild conditions with diethyl acetonedicarboxylate to yield the enamine precursor to the actual cyclization step in the Pictet-Spengler reaction. This enamine could be isolated and purified by chromatography on a large scale and in excellent yields. The enamine, neat, was then treated with dry trifluoroacetic acid at low temperature to effect cyclization, again in excellent yield: wet trifluoroacetic acid lead to hydrolysis of the enamine, and decreased yields. Cyclization under these conditions lead to exclusive formation of the endo product.



The diester was then elaborated into the diol **8** via a two step methylation and reduction sequence. As mentioned before, several attempts to halogenate this species lead to exclusive pyran formation. Eventually, we found that treatment of this diol with thionyl chloride in methylene chloride, yielded the hydrochloric acid salt of the bis-chloro analog of **8** in nearly quantitative yield.[19]

Synthesis of Class IIA: Integrated Steroids

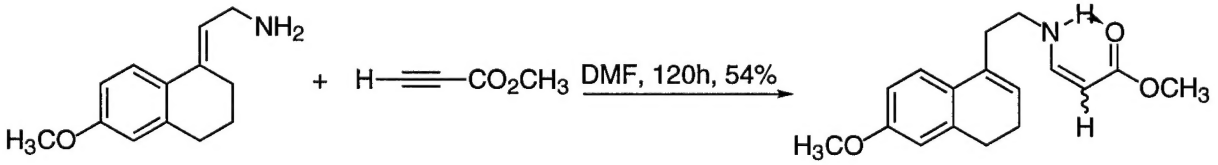
Although not presented in the original proposal, the synthesis of the class IIA compounds parallels that of class IA: the synthetic transformations are the same with the exception of substituting 3-hydroxypropionaldehyde for diethyl acetonedicarboxylate in the Pictet-Spengler cyclization.

The preparation of unprotected 3-hydroxypropionaldehyde proved to be a tedious task. Instead, we prepared several protected forms of 3-hydroxypropionaldehyde easily from 1,3-

propanediol via a monoprotection and Swern-type oxidation sequence. Cyclization with these aldehydes proved to be unsuccessful under the conditions discussed above for the formation of piperidines: aldol reactions and β-elimination of the alkoxy substituent prevented efficient cyclization. We decided to instead use an oxidized form, ethyl 3-oxo-propionate, as a reactant in the cyclization. Ethyl 3-oxo-propionate was prepared simply by ozonolysis of ethyl but-3-enoate. Once again, aldol reactions were a problem under the acidic conditions of the cyclization, and we decided to seek an alternative route.

We had previously known that ethyl glyoxylate underwent the Pictet-Spengler reaction efficiently. This sequence left us with an α-amino ester which could be homologated under standard Arndt-Eistert conditions to the desired β-amino ester.[20] Although we achieved the successful synthesis of the homologated compound, the overall route was somewhat cumbersome: we still had to separate mixtures of olefin isomers following the cyclization reaction.

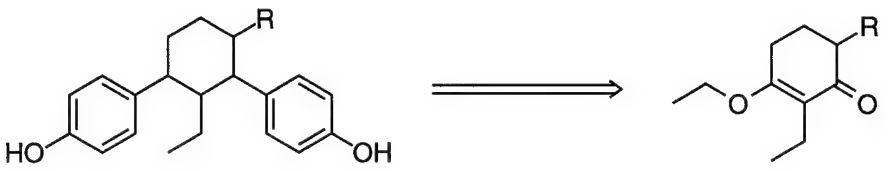
In the literature it was known that amines would add to activated alkynes, in a 1,4 fashion to form stabilized enamines under mild conditions.[21] We applied this knowledge to our system, and successfully added the primary amine derived above to methyl propiolate to yield a mixture of enamine isomers which are the precursors to the cyclization reaction. The ratio of the enamine isomers was 2:1 in favor of the Z-isomer: which may be explained by an intramolecular hydrogen-bond between the amine and the ester carbonyl: visible in the ^{1}H NMR spectrum of the compound.



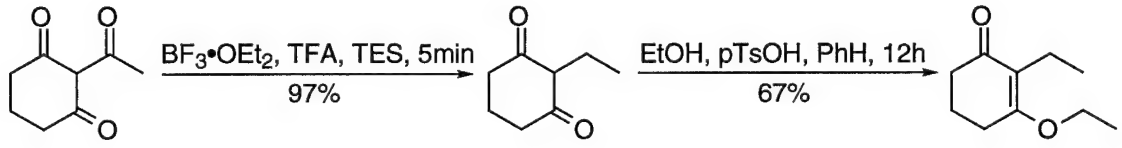
The mixture of enamine isomers could then be treated at low temperature with neat trifluoroacetic acid, as before, to effect cyclization to a racemic mixture of the endo olefin isomer exclusively. Reduction of the ester was then achieved readily under standard conditions, followed by bromination with carbon tetrabromide and triphenylphosphine

Synthesis of Class IB: A Carbocyclic Analog of Benzestrol

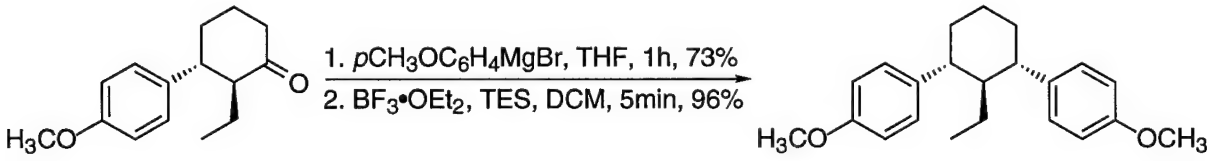
Because they are the most unusual structurally, we attempted to make a model compound of the class IIB series before beginning work on the actual targets. The cyclohexane core of these model compounds is very versatile structurally, and we intended to exploit this to probe for the optimal design of class IIB.



Access to the properly functionalized cyclohexane core was afforded by a two-step sequence of deoxygenation and protection of commercially available 2-acetyl-1,3-cyclohexadione.[22] This route proved superior to the rather difficult alkylation of 1,3-cyclohexadione itself: this route would be used to further probe the role of the ethyl group in ER binding.



Addition of the appropriate aromatic Grignard reagent followed by dissolving metal reduction of the enone, afforded the substituted cyclohexanone in good yield. Subsequent addition of another aromatic Grignard reagent to the ketone, followed by deoxygenation and deprotection of the resulting benzylic alcohol[23] afforded the desired bis-phenol as a mixture of isomers.



Binding affinity analysis on this mixture reveals that indeed there is moderate binding affinity for the ER. We were encouraged by this result, and efforts continue within this series of compounds. The class IIB compounds therefore appear viable, and we intend to synthesize several new analogs in the near future.

Progress in Relation to the Statement of Work

The complete three year Statement of Work, presented in the original proposal of July 1997, is shown below:

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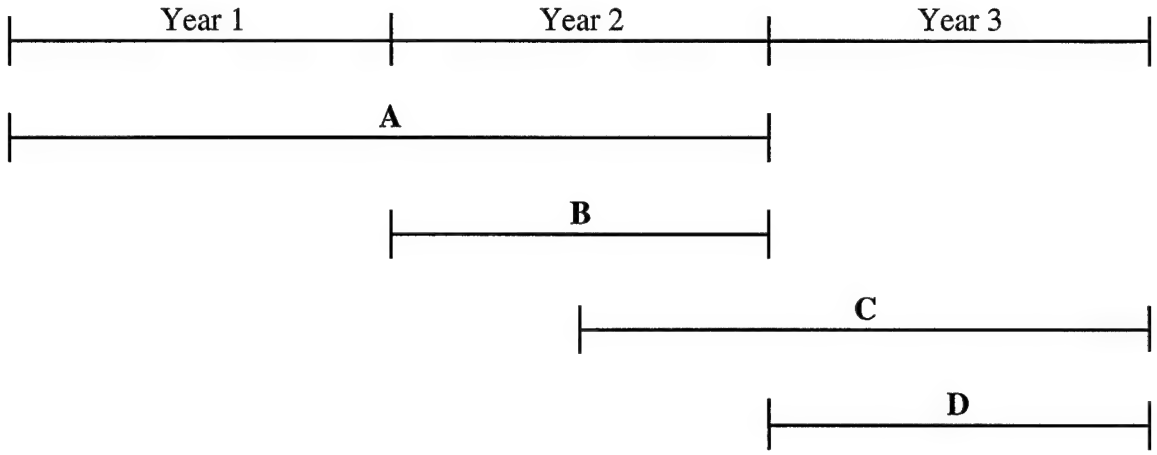
ORIGINAL STATEMENT OF WORK

Project Period: July 1, 1997– June 30, 2000 (3 years)



Prepare nonradioactive Ga compounds whose structures favor binding with the human estrogen receptor (ER).

- **Task A:** Months 1-24: Develop syntheses for the proposed Receptor-Based Imaging Agents (RBIA's) using non-radioactive ^{69}Ga.

Determine in vitro the estrogenicity of each Ga compound by measuring its Receptor Binding Affinity (RBA) and its non-specific binding.

- **Task B:** Months 13-24: The specific binding of nonradioactive RBIA's to ER will be measured by the standard Receptor Binding Affinity (RBA) assay.

Prepare radiolabeled samples of Ga-containing estrogens that have high RBAs and test their in vitro stability.

- **Task C:** Months 19-36: Synthesis of RBIA's will be achieved by repeating the most efficient techniques from Task A with ^{67}Ga or ^{68}Ga.

Evaluate radiolabeled Ga-containing estrogens as imaging agents for breast tumors.

- **Task D:** Months 25-36: Through our long-standing collaboration with Professor Michael Welch of the Mallinckrodt Institute of Radiology at Washington University Medical School we will evaluate the *in vivo* tissue distribution of RBIA's with promising *in vitro* properties.

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It is evident from the results presented in the preceding sections that we have progressed quite rapidly through the syntheses of classes IA and IIA, as outlined in the Statement of Work for Year 1. In addition, we are already progressing on elements of the Statement of Work for Year 2, albeit on carbocyclic analogs of the target RBIA's. We are also beginning preliminary work on simple intramolecularly-stabilized gallocycles as models for tracer-level syntheses.

CONCLUSIONS

From the results we have achieved so far, we have shown that the proposed synthetic routes are indeed viable methods for making the cores of these novel imaging agents. The design of these cores has been shown to be reasonable given the lead RBA of the synthetic carbocyclic analog. Thus the project is progressing well along the lines initially envisioned in the original proposal.

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Table 1. Comparison of Ga Compounds with Requirements of Ga-Containing Estrogen – From the Original Proposal

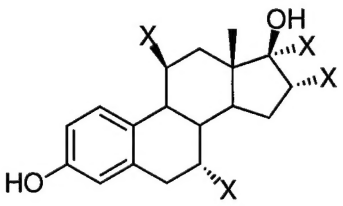
Property	Ideal Ga-Containing Estrogen	Ga Coordination Complexes	Ga Organometallics[1]
Ga Coordination Number	≤4	6	4
Stability in Aqueous Environment	high	high	high
Solubility at Physiological pH	high	variable[2]	high
Binding or Release of Ga to Transferrin	none	variable[2]	weak

1. Represented by the only such compound evaluated for all four properties: $(CH_3)_2Ga(C_5H_7O_2)$
2. May be low or high, depending on the presence of stabilizing ligands for Ga

Figure 1. Structural Diversity of Estrogens and Ga-Containing Analogs – From the Original Proposal

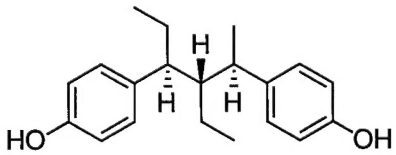
A. Estrogen

Steroidal



A. Estradiol

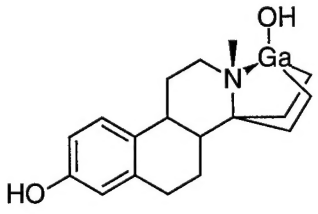
Three-Carbon Bridged



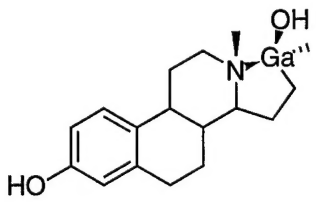
B. Benzestrol

B.Targeted Ga-Containing Analog

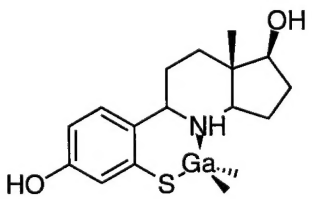
Steroidal Integrated



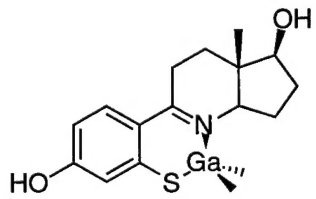
IA



IIA

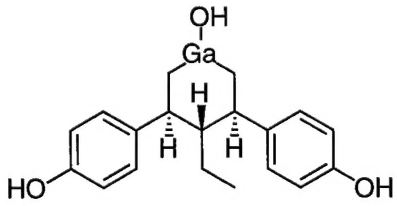


IIIA



IVA

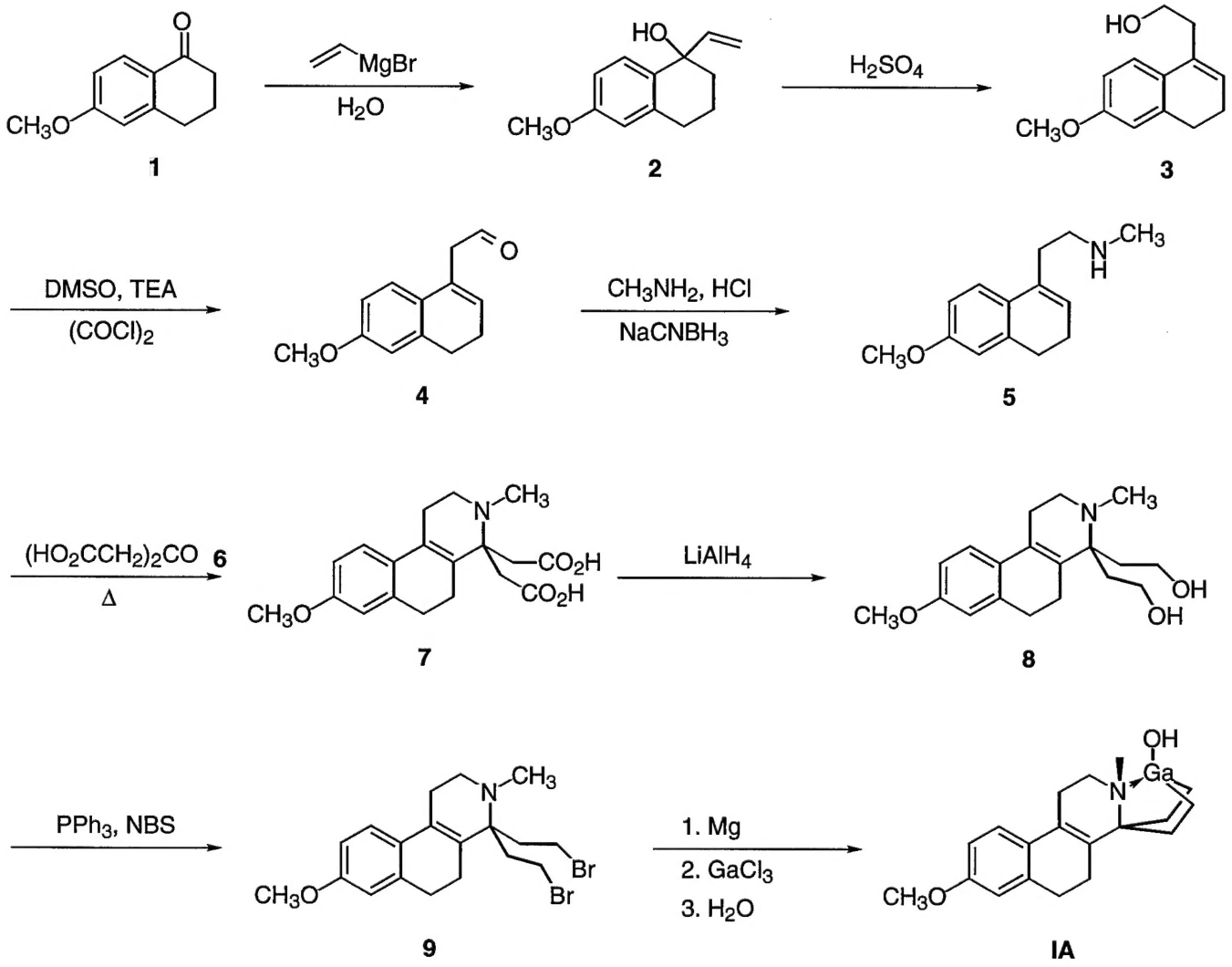
Nonsteroidal Conjugated



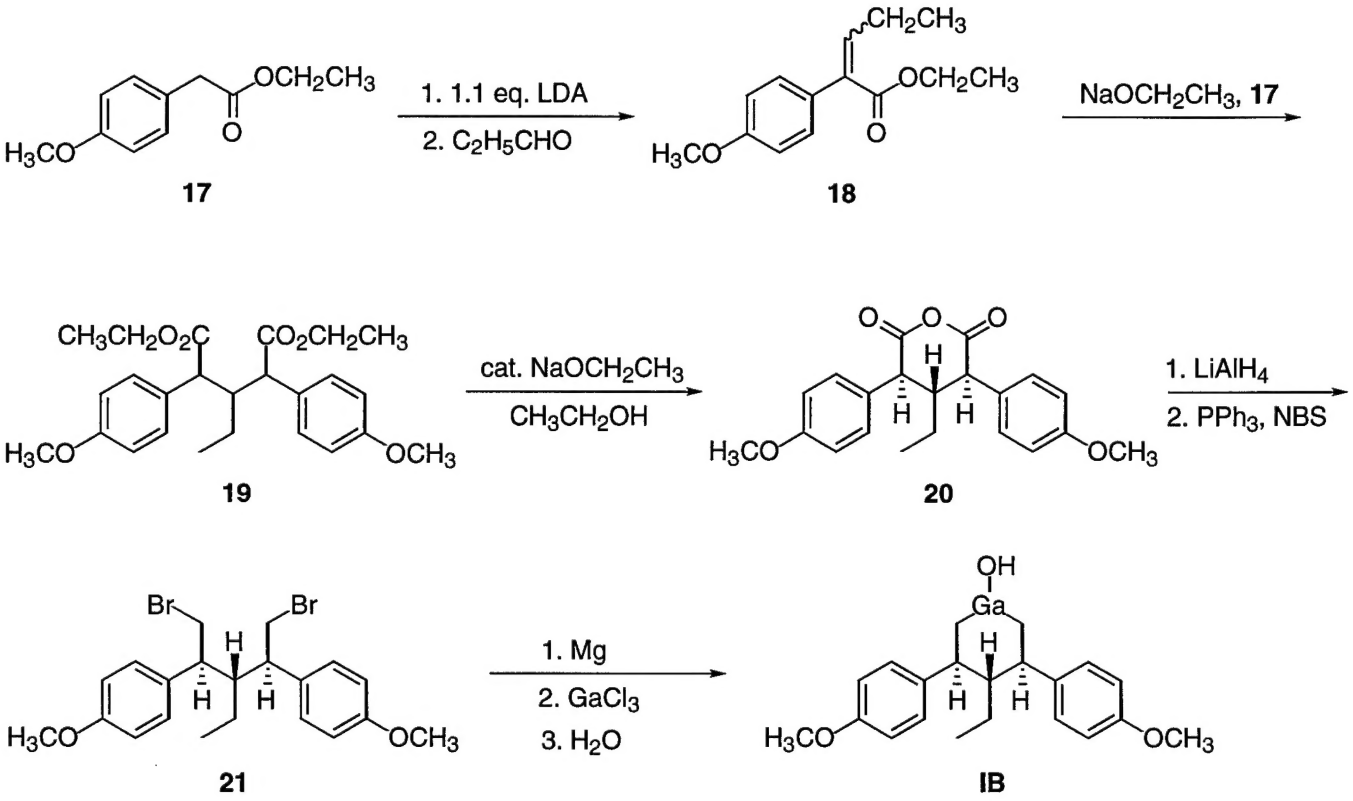
IB

X indicates a site where a large group may be placed without affecting affinity for ER

Scheme 1. Proposed Synthesis of Class IA – From the Original Proposal



Scheme 2. Proposed Synthesis of Class IB – From the Original Proposal



17 **18** **19** **20** **21** **IB**